Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration

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The terminal differentiation of myelinating glia involves complex interactions that culminate in the formation of myelin. The POU domain transcription factor Tst-1/Oct-6/SCIP is expressed transiently during myelination, and we report here that it has a critical role in this developmental process. Deletion of the *Tst-1/Oct-6/SCIP* gene produces a severe defect in peripheral myelination by arresting Schwann cell maturation before axonal wrapping. Unexpectedly, the activation of major myelin-specific genes appears to be unaffected by the *Tst-1/Oct-6/SCIP* mutation, demonstrating that multiple, independently regulated events are required for terminal differentiation of Schwann cells. In addition, aberrant differentiation and migration of specific neurons in *Tst-1/Oct-6/SCIP* mutant homozygotes is associated with a fatal breathing defect, providing a model for investigating the regulation of pulmonary homeostasis.

[**Key Words:** POU domain transcription factor; peripheral myelination; respiration; Tst-1; Oct-6; SCIP; nucleus of the lateral olfactory tract; phrenic nucleus; neuronal migration; Schwann cell]

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Tst-1/Oct-6/SCIP is a member of the POU domain family of transcriptional regulators initially defined by the isolation of genes that encode the pituitary-specific transcription factor Pit-1/GHF-1 (Bodner et al. 1988; Ingraham et al. 1988), the octamer-binding proteins Oct-1 (Sturm et al. 1988) and Oct-2/OTF-2 (Clerc et al. 1988; Ko et al. 1988; Müller et al. 1988; Scheidereit et al. 1988), and by characterization of the *Caenorhabditis elegans* *Unc-86* mutation (Finney et al. 1988). This family of proteins is characterized by its possession of a POU-specific DNA-binding domain, which is structurally similar to λ repressor, tethered to a variant homeo domain (for review, see Herr et al. 1988; Verrijer and Van der Vliet 1993; Wegner et al. 1993a; Herr and Cleary 1995). The large number of POU domain transcription factors has been classified into six groups based on structural homologies (for review, see Verrijer and Van der Vliet 1993; Wegner et al. 1993a). Tst-1/Oct-6/SCIP is a member of the Class III POU domain proteins, which are expressed primarily in the developing and mature nervous system (He et al. 1989).

The *Tst-1/Oct6/SCIP* gene was characterized independently from rat testis and brain (He et al. 1989, 1991),

rat sciatic nerve (Monuki et al. 1989), F9 cells (Suzuki et al. 1990), mouse brain (Meijer et al. 1990), and epidermal keratinocytes (Faus et al. 1994). The *Tst-1/Oct6/SCIP* gene (*Otf-6*) is located on mouse Chromosome 4 (Rohdewohld and Gruss 1992; Avraham et al. 1993), and is intronless; it appears to have evolved by retrotransposition (Kuhn et al. 1991). In the early mouse embryo, Tst-1/Oct-6/SCIP expression undergoes two waves of induction and restriction, one in primitive ectoderm and one in primitive brain (Zwart et al. 1996). Later, it is expressed in multiple neuronal populations in the brain (He et al. 1989; Franz et al. 1994; Alvarez-Bolado et al. 1995). It is also expressed in embryonic stem (ES) cells (Meijer et al. 1990; Suzuki et al. 1990), testis (He et al. 1989), and epidermis (He et al. 1989; Faus et al. 1994). In the sciatic nerve, Tst-1/Oct6/SCIP is transiently expressed. Maximal expression occurs before the peak of myelin gene expression (Monuki et al. 1990; Scherer et al. 1994). In cultured Schwann cells, Tst-1/Oct-6/SCIP expression is up-regulated by axons (Scherer et al. 1994) and agents that raise the concentrations of intracellular cAMP (Monuki et al. 1989). In addition to its expression in myelinating Schwann cells, the *Tst-1/Oct-6/SCIP* gene is expressed at specific stages in the maturation of oligodendrocytes (Collarini et al. 1991, 1992).

The Tst-1/Oct-6/SCIP protein can function either as an activator of transcription or as a repressor. In tran-

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sient cotransfection assays, *Tst-1/Oct-6/SCIP* activates octamer-containing promoters (Suzuki et al. 1990; Meijer et al. 1992; Monuki et al. 1993b; Renner et al. 1994), the promoter for the acetylcholine receptor $\alpha 3$ subunit (Yang et al. 1994), and the JC virus early and late promoters (Wegner et al. 1993b; Renner et al. 1994), but represses the promoters of genes that encode the myelin structural protein P_0 (Monuki et al. 1990, 1993a,b; He et al. 1991), myelin basic protein (MBP; Monuki et al. 1990), nerve growth factor receptor (Monuki et al. 1990), and the keratin K5 and K14 promoters (Faus et al. 1994). An effector domain in the amino terminus (Meijer et al. 1992; Monuki et al. 1993a,b) is required for transactivation or repression and can act in a cell type-specific manner (Monuki et al. 1993b); maximal activation or repression, however, requires both the amino terminus and the POU domain. In addition to binding DNA, the POU domain acts also as a scaffold for protein-protein interactions [for review, see Herr and Cleary 1995]; the POU domain of the *Tst-1/Oct-6/SCIP* protein interacts with the viral large T/small t antigens (Renner et al. 1994) and the non-histone chromatin proteins HMG-I/Y (Leger et al. 1995). These interactions suggest that *Tst-1/Oct-6/SCIP* may function as part of cell type-specific complexes of proteins that can activate or repress genes.

Myelin, a structure required for rapid axonal conduction, is produced by oligodendrocytes in the central nervous system (CNS), and by Schwann cells in the peripheral nervous system (PNS). The Schwann cell lineage progresses through defined developmental stages from neural crest to produce myelinating and nonmyelinating Schwann cells [for review, see Jessen and Mirsky 1991, 1992; Eccleston 1992]. Axons produce factors that promote the proliferation and maturation of precursor Schwann cells (Jessen et al. 1994; Dong et al. 1995; Gavrilovic et al. 1995). Axonal contact stimulates the differentiation of Schwann cells, perhaps in part through elevation of intracellular cAMP levels (Morgan et al. 1991; for review see Mezei 1993). After rapid proliferation and invasion of bundles of axons, myelinating Schwann cells cease cell division, then ensheath and wrap large caliber axons [for review, see Webster 1993; Hildebrand et al. 1994], whereas nonmyelinating Schwann cells associate with small caliber axons, and retain many of the characteristics of immature Schwann cells. Based on evidence from transient cotransfection and transgenic mouse studies, it has been proposed that *Tst-1/Oct-6/SCIP* serves as a repressor of myelination in developing Schwann cells (Monuki et al. 1990, 1993a,b; Weinstein et al. 1995).

In this paper we report that disruption of the *Tst-1/Oct-6/SCIP* gene results in defects of both neurons and peripheral myelinating glia. In the absence of *Tst-1/Oct-6/SCIP*, mice exhibit a striking absence of peripheral myelin. Most myelinating Schwann cells appear to be arrested at the promyelinating stage, in which they have ensheathed a single axon in a 1:1 manner but have not yet formed a myelin sheath (Webster 1993). These arrested Schwann cells seem to express correctly several myelination-related transcripts, including mRNAs for P_0

and MBP. Central myelination by oligodendrocytes appears to proceed normally. The phenotype of *Tst-1/Oct-6/SCIP* mutant mice redefines the critical role of this transcription factor in the maturation of myelinating Schwann cells and provides new insights into the complex regulatory mechanisms that control myelination. Furthermore, *Tst-1/Oct-6/SCIP* gene deletion results in a fatal defect in breathing that is distinct from its myelination phenotype and is associated with specific neuronal abnormalities.

Results

Disruption of the Tst-1/Oct-6/SCIP gene

To determine the role that *Tst-1/Oct-6/SCIP* has in the development of the cell types in which it is expressed, mice that lack the majority of *Tst-1/Oct-6/SCIP*-coding sequences were generated using (ES) cells. Targeted replacement of the entire amino terminus and POU domain with a functional β -galactosidase transcription unit (*lacZ* gene; Fig. 1a,b) resulted in a null mutation that allowed the fate of cells expressing the *Tst-1/Oct-6/SCIP* promoter to be evaluated by X-gal staining. Heterozygous mice displayed no overt phenotype. Approximately one quarter of the pups born from heterozygous intercrosses were mutant (Fig. 1b), indicating their embryonic viability. Homozygous mutant mice lacked *Tst-1/Oct-6/SCIP* protein (Fig. 1c), confirming that the gene was mutated, and the pattern of β -galactosidase expression in heterozygous animals accurately reflected that of the endogenous *Tst-1/Oct-6/SCIP* gene throughout development (Fig. 1d). *Tst-1/Oct-6/SCIP* ($-/-$) pups were usually cyanotic at birth and most died within 2 hr. However, rare ($-/-$) mice appeared to breathe normally, and survived up to 22 days, although they were runted severely. These "outliers" were observed only among pups that possessed both 129/Sv and C57BL/6 genetic backgrounds (data not shown). No defects were noted in the skin, where *Tst-1/Oct-6/SCIP* is expressed strongly (He et al. 1991; Faus et al. 1994).

Arrest of peripheral myelination in Tst-1/Oct-6/SCIP mutant mice

To determine whether *Tst-1/Oct-6/SCIP* is essential for myelination in the CNS and PNS, we examined semithin sections of the spinal cord and sciatic nerve of *Tst-1/Oct-6/SCIP* ($-/-$), ($+/-$; data not shown), and ($+/+$) mice at postnatal day 3 (P3). In the spinal cord, there were comparable numbers of myelinated axons in the ventral funiculus in mice of all three genotypes, indicating that *Tst-1/Oct-6/SCIP* is not essential for the development of mature oligodendrocytes, or the formation of their myelin sheaths (Fig. 2a,b). In contrast, there were dramatically fewer myelinated axons in the sciatic nerves of *Tst-1/Oct-6/SCIP* ($-/-$) mice as compared with their ($+/-$) and ($+/+$) littermates (Fig. 2c,d). These results indicate that *Tst-1/Oct-6/SCIP* is required for the development of myelinating Schwann cells.

We examined P0 (day of birth) to P5 sciatic nerves by

Figure 1. Disruption of the *Tst-1/Oct-6/SCIP* gene. **(a)** The targeting vector is shown undergoing homologous recombination with the wild-type *Tst-1/Oct-6/SCIP* locus, replacing the entire amino terminus and POU domain coding regions of *Tst-1/Oct-6/SCIP* with the *lacZ* gene and an MC1-*neo* expression cassette. Parentheses indicate restriction sites that were destroyed during construction of the targeting vector. Probes used to analyze homologous recombination are shown below the mutant locus, as are the wild-type and mutant RFLPs that are obtained when DNA from ES cells or mice is digested with *Bgl*II and *Eco*RV. **(b)** 12.7-kb wild-type and 9.0-kb mutant RFLPs from three independent ES cell lines and from F₂ mice that result from hybridization to the 3' probe. **(c)** Tst-1/Oct-6/SCIP protein is not detected in (-/-) mice by an anti-Tst-1/Oct-6/SCIP antiserum. Layer V neurons are shown in transverse sections of cerebral cortex from P0 wild-type (+/+) and mutant (-/-) pups. Tst-1/Oct-6/SCIP-immunoreactive nuclei are absent from the (-/-) P0 brain. Scale bar, 63 μ m. **(d)** Anti-Tst-1/Oct-6/SCIP labeling of (+/+) cortex, and β -galactosidase staining of (+/-) cortex, produce similar patterns of cortical layers II/III and V, and the CA1 region of the hippocampus. Both sections are from 3-week-old mice; the antibody section is transverse, whereas the β -galactosidase section is sagittal.

electron microscopy, to further evaluate the morphological defect in *Tst-1/Oct-6/SCIP* (-/-) mice. At P0, Schwann cells in (-/-), (+/-), and (+/+) mice appeared similar, having reached the promyelinating stage of their development; few, if any, myelin sheaths were seen. By P3 (Fig. 3a,b), the vast majority of axons in *Tst-1/Oct-6/SCIP* (-/-) mice were unmyelinated, as compared with their (+/+) and (+/-) littermates. In the (-/-) mice, most myelinating Schwann cells failed to mature beyond the 1:1 ensheathment stage (Webster 1993), resulting in more Schwann cells in this configuration than in their (+/+) and (+/-) littermates. A few myelin sheaths that were found, however, that had a normal ultrastructure (Fig. 3c,d). Therefore, Tst-1/Oct-6/SCIP is required for the maturation of myelinating

Schwann cells. Because Tst-1/Oct-6/SCIP expression correlates with Schwann cell proliferation in developing and transected nerves (Monuki et al. 1990), we counted the number of Schwann cell nuclei on low-magnification electron micrographs in P3 (-/-) and (+/+) animals. The number of Schwann cells was comparable in both, indicating that no defect occurred either in the proliferation of Schwann cells or in their exit from the cell cycle. We examined the basal laminae in (-/-) and (+/+) nerves, because Schwann cells require an intact basal lamina for axonal wrapping (for review, see Bunge et al. 1993) as demonstrated by the defective myelination in laminin α 2-deficient *dy/dy* mice (Stirling 1975; Weinberg et al. 1975; Xu et al. 1994). There were well-developed basal laminae surrounding every Schwann cell (ar-

Figure 2. Myelination in the CNS and PNS of *Tst-1/Oct-6/SCIP* (-/-) mice. These photomicrographs of transverse, semi-thin sections of spinal cord (a,b) and the sciatic nerve (c,d) of a P3 (+/+) (a,c) and (-/-) littermate (b,d) demonstrate that myelination is impaired severely in the sciatic nerve, but not in the ventral funiculus in the (-/-) animal. A single myelinated axon in d is indicated by an arrow. Scale bar, 10 μ m.

rows, Fig. 3c,d), therefore the failure of myelinating Schwann cells to develop in (-/-) mice cannot be attributed to the absence of basal laminae.

Myelination-specific gene expression in *Tst-1/Oct-6/SCIP* mutant mice

To determine the effects of the *Tst-1/Oct-6/SCIP* mutation on the expression of specific Schwann cell proteins, antisera directed against these proteins were used for immunohistochemical analysis of P4 (+/-), (-/-) and (+/+; data not shown) sciatic nerves. Normal laminin immunoreactivity around (-/-) Schwann cells (Fig. 4a), provided further evidence that their basal laminae were unaffected by the mutation. P_0 protein was nearly absent in the mutant, except for occasional P_0 -positive myelin sheaths (Fig. 4b), which corroborated the observation of a few myelinated axons in (-/-) nerves. MBP immunoreactivity was similar to that for P_0 (data not shown). In contrast, both MAG and periaxin immunoreactivity were found around many axons in the mutant (Fig. 4c,d).

As both of these proteins have been shown to be localized to the adaxonal surface of myelinating Schwann cells in developing nerves (Gillespie et al. 1994), these data indicate that mutant Schwann cells not only ensheath axons in the expected 1:1 manner, but also express MAG and periaxin, and correctly target them to their adaxonal surface. Therefore, even though these mutant Schwann cells fail to elaborate a myelin sheath, they possess many of the characteristics of initially myelinating Schwann cells.

We examined the mRNAs from several genes that are expressed during Schwann cell differentiation, to investigate further whether the expression of these genes is affected by the *Tst-1/Oct-6/SCIP* deletion. *Tst-1/Oct-6/SCIP* had been thought previously to act as a repressor of P_0 and MBP gene expression (Monuki et al. 1990; He et al. 1991; Weinstein et al. 1995). However, P_0 , MBP, and MAG expression signals were comparable in mutant and

Figure 3. Myelination is deficient in *Tst-1/Oct-6/SCIP* mutant mice. (a) Wild-type. (a) Representative axons that have been ensheathed by Schwann cells in a 1:1 manner. Arrowheads mark bundles of unmyelinated axons. These electron micrographs demonstrate a lack of myelinated axons in *Tst-1/Oct-6/SCIP* (-/-) mice (b) as compared with (+/+) mice (a). One of the few myelinated axons found in the (-/-) mouse has a normal basal lamina (arrowheads in d) and myelin sheath (inset), with normal periodicity (15 nm) of the major dense lines (arrowheads); compare with the myelinated fiber in c, from a normal littermate. Scale bars, 5 μ m (a,b); 0.5 μ m (c,d).

Figure 4. Myelin-specific protein expression in developing wild-type and mutant sciatic nerves. Adjacent sections from P4 (+/-) and (-/-) nerves were labeled for laminin (a), P₀ (b), MAG (c), and periaxin (d) protein. There were many fewer P₀-positive myelin sheaths in the (-/-) nerve as compared with the (+/-) nerve, whereas the number of MAG- and periaxin-immunoreactive Schwann cells were comparable. Although the apparent reduction in MAG and periaxin immunofluorescence in (-/-) nerves may reflect the absence of Schmidt-Lanterman incisures and/or decreased expression of these proteins, both proteins are correctly targeted to the adaxonal membrane. Scale bar, 10 μ m.

wild-type mice, both at P0 and at P4 (Fig. 5a–c), and comparable levels of P₀ mRNA were observed in P10 sciatic nerves from (+/+) and (-/-) mutant mice (Fig. 5b). In addition, no differences in PMP-22 expression were observed (data not shown). Although in situ hybridization may not detect small differences in mRNA levels, these results strongly suggest that Tst-1/Oct-6/SCIP does not regulate directly the expression of these genes in vivo. The presence of normal levels of P₀ mRNA but reduced levels of P₀ protein in the (-/-) sciatic nerves is consistent with the observation that Schwann cells shunt P₀ protein to the lysosome under conditions that prevent myelination (Brunden and Poduslo 1987). p75 nerve growth factor receptor (NGFR) is down-regulated as Schwann cells differentiate (Morgan et al. 1991) and

the normal pattern of p75 NGFR expression in mutant sciatic nerve (Fig. 5d) further indicates that mutant Schwann cells have initiated their differentiation toward a myelinating phenotype. Together, the immunohistochemistry and in situ hybridization results demonstrate that Tst-1/Oct-6/SCIP probably functions independently of the activation of the major myelin genes, indicating that multiple genetic pathways control myelination.

Neuronal defects in Tst-1/Oct-6/SCIP (-/-) mice

The respiratory failure in Tst-1/Oct-6/SCIP mutant mice may result from abnormal neuronal control of breathing and ventilatory drive, and/or failure of lung

Figure 5. In situ hybridization to sections from sciatic nerve of P0 (+/-) and (-/-) littermates, and of P4 and P10 (+/+) and (-/-) littermates, with antisense strand probes for P₀ (a), MAG (b), MBP (c), and p75 NGFR (d) demonstrates that the major myelin mRNAs are present at normal or near-normal levels in Tst-1/Oct-6/SCIP (-/-) mice, both at the onset of myelination (P0), and after myelination has begun (P4, P10). Examination of the contralateral sciatic nerves from the P4 mice confirmed that the mutant nerve was unmyelinated, in contrast to the control nerve (data not shown). No signal was observed on adjacent sections of P0 and P4 sciatic nerve that were hybridized with P₀ and MAG sense strand probes (data not shown).

gas exchange. This phenotype is unlikely to result from hypomyelination, because little or no myelination has occurred at birth and mutations in other genes that disrupt myelination do not result in these respiratory defects (Snipes and Suter 1995). Homozygous mutant pups exhibited variable degrees of disordering of the phrenic motoneurons and a possible decrease in their number (Fig. 6a,b), suggesting improper control of the diaphragm. In addition to its expression in phrenic motoneurons, *Tst-1/Oct-6/SCIP* also is expressed in many nuclei required for respiratory rhythm and airway caliber (Duffin et al. 1995; Feldman et al. 1995), including the nucleus tractus solitarius, the nucleus ambiguus, the glossopharyngeal nucleus, and the facial nucleus (Fig. 6c,d). Although most mutant pups died too quickly to permit a meaningful respiratory analysis, four mutant pups that survived for several hours displayed a significant reduction in breathing frequency when compared with 10 homozygous wild-type littermates (69 ± 7 SEM vs. 107 ± 8 SEM; unpaired *t*-test, $P = 0.013$; Fig. 6e). Heterozygous pups displayed an intermediate frequency (91 ± 9 SEM, $n = 17$). Breathing was more irregular in mutant pups, and tended to occur in periodic bouts with long periods of apnea between bouts. The differences between the surviving homozygous mutant pups and homozygous wild-type pups in tidal volume per gram of body weight, or in inspiratory volume per gram of body weight, were not significant. There were no obvious differences between the mutants studied and their wild-type littermates in the magnitude of the ventilatory response to acute (30 sec) or sustained (3 min) hypoxia, the ventilatory decline during sustained hypoxia or on restoration of normoxia after sustained hypoxia, or the ventilatory response to hypercapnia (data not shown). Although no gross anatomical differences were observed in lungs from mutant and wild-type pups, the results of histological examination of lung sections from several moribund neonatal mutant pups (data not shown) suggests that improper lung inflation may cause the respiratory failure of the mutant pups that die immediately after birth. *Tst-1/Oct-6/SCIP* expression has not been observed in lung at e13 d.p.c. (days post-coitum) or e16 d.p.c. in rat (He et al. 1989) or at birth in mouse (data not shown), suggesting that the effect of the *Tst-1/Oct-6/SCIP* mutation in lung is non-cell autonomous.

Although most brain regions in homozygous *Tst-1/Oct-6/SCIP* ($-/-$) mice were structurally indistinguishable from those of heterozygous mice, staining whole brains for β -galactosidase revealed a defect in the nucleus of the lateral olfactory tract (NLOT; Fig. 6f–j). At E18.5, NLOT in heterozygotes is near the ventral surface of the brain, whereas in mutants, putative NLOT cells are located in the interior of the brain (Fig. 6f). In P0 heterozygous brains, NLOT can be observed in its normal position, posterior to the lateral cerebral sulcus that separates the lateral olfactory tract from the olfactory tubercle. In P0 ($-/-$) brains, NLOT is consistently missing from its normal location, and is often replaced by a new, dispersed patch of β -galactosidase-positive cells appearing medial and anterior to the lateral cerebral

sulcus (Fig. 6g). Additional patches of abnormal cells are occasionally seen. At P4, the displaced ($-/-$) NLOT is smaller than the ($+/-$) NLOT, or is absent altogether. Occasional abnormal axon tracts can be observed emanating from the vicinity of the ($-/-$) NLOT (Fig. 6h). By P10, no staining of NLOT cells can be observed in ($-/-$) brains (Fig. 6i). The locations in P0 and P4 brains of normal and putative displaced NLOT cells are diagrammed in Figure 6j. The most likely interpretation of these data is that during development, neurons of NLOT migrate to their final position, and that this migratory event, and the subsequent survival of these neurons, requires expression of *Tst-1/Oct-6/SCIP*.

Discussion

Role of Tst-1/Oct-6/SCIP in Schwann cell differentiation

The phenotype of *Tst-1/Oct-6/SCIP* mutant mice reveals that this POU domain factor exerts a critical regulatory function in peripheral myelination. The maturation of myelinating Schwann cells and the pattern of *Tst-1/Oct-6/SCIP* expression during myelination are summarized in Figure 7. In Schwann cells, *Tst-1/Oct-6/SCIP* expression is transient, probably activated by axonally induced increases in levels of intracellular cAMP (Monuki et al. 1989; Scherer et al. 1994); *Tst-1/Oct-6/SCIP* expression declines as myelin sheath formation commences (E. Arroyo, J. Bermingham Jr., M.G. Rosenfeld, and S. Scherer, unpubl.). Deletion of *Tst-1/Oct-6/SCIP* does not affect the number of Schwann cells, indicating that no proliferative defect has occurred. *Tst-1/Oct-6/SCIP* mutant Schwann cells possess a unique phenotype that exhibits many of the characteristics of myelinating Schwann cells. They form 1:1 associations with axons, produce a normal basal lamina, and properly localize MAG and periaxin to the adaxonal membrane. Although the *Tst-1/Oct-6/SCIP* mutation does not appear to affect the expression of myelin structural genes, P_0 and MBP protein expression is decreased, and axonal wrapping is disrupted. Wrapping is not critically dependent on the major myelin proteins because Schwann cells from mice that lack P_0 (Giese et al. 1992), MAG (Montag et al. 1994), PMP22 (Adlkofer et al. 1995), or both P_0 and MBP (Martini et al. 1995) are able to progress beyond the ensheathment stage. Therefore, the targets of *Tst-1/Oct-6/SCIP* regulation are critical regulators of axonal wrapping.

Deletion of *Tst-1/Oct-6/SCIP* in mice has resolved conflicting views of its potential role in the activation or repression of myelin genes. Previously, *Tst-1/Oct-6/SCIP* had been postulated to function in myelination by repressing P_0 and MBP gene expression, based on its repression of the P_0 and MBP promoters in transient cotransfection studies (Monuki et al. 1989 1993a,b), and on the actions of Δ SCIP, a putative dominant-negative truncated form of *Tst-1/Oct-6/SCIP*, which retains the POU domain and carboxyl terminus (Weinstein et al. 1995). Δ SCIP precociously activates myelination when ex-

Figure 6. Neuronal defects in *Tst-1/Oct-6/SCIP* mutant mice. (a) β-galactosidase stained cervical spinal cord of heterozygous newborn mouse. Cervical spinal nerves (C₁–C₆) are visible because of the staining of Schwann cells, and the phrenic nuclei are located bilaterally between C₃ and C₆. (b) Disorganization of phrenic nuclei from homozygous mutant newborn mouse is revealed by β-galactosidase staining. (c) In situ hybridization of a *Tst-1/Oct-6/SCIP* probe to a transverse section through a P0 (+/+) brainstem, showing nucleus tractus solitarius (NTS), glossopharyngeal (XII) nucleus, and nucleus ambiguus (NA). (d) In situ hybridization of a *Tst-1/Oct-6/SCIP* probe to a transverse section through a P0 (+/+) brainstem, showing facial (VII) nucleus. (e) Results of plethysmograph recordings of P0 (+/+) and (-/-) mice, showing reduced frequency of breathing in (-/-) mice. Because the (-/-) pups tended to gasp, and gasps were not distinguished from normal breaths in the analysis of respiratory rate, the actual respiratory rate may actually be lower in mutant pups than we have estimated. (f) Heterozygous (+/+) and homozygous mutant (-/-) E18.5 brains stained for β-galactosidase, and cleared by infusion with 30% sucrose. NLOT refers to the nucleus of the lateral olfactory tract, which is replaced in the (-/-) brain by novel β-galactosidase-expressing cells (large arrowhead) at a more internal position in the brain. (g) P0 (+/+) and (-/-) brains stained for β-galactosidase. Putative displaced NLOT cells (large arrowhead) can be observed in a more medial and anterior position in the (-/-) brain. Additional abnormal β-galactosidase staining cells are occasionally seen, and are marked with a small arrowhead. Scale bar, 1 mm. (h) P4 (+/+) and (-/-) brains. Novel β-galactosidase-stained cells and axon tracts in the (-/-) brain are marked with large and small arrowheads, respectively. Scale bar, 1 mm. (i) P10 (+/+) and (-/-) brains stained for β-galactosidase. NLOT cells are missing in the (-/-) brain. Note also the disappearance of β-galactosidase staining in the facial (VII) nucleus in both (+/+) and (-/-) brainstems, suggesting that *Tst-1/Oct-6/SCIP* expression in these cells is transient. (j) Diagram depicting NLOT at P0 and P4. The broken lines represent the lateral cerebral sulcus, and the relative locations of normal and mutant NLOT cells (●) have been exaggerated for clarity. The broken open circles in the P4 (-/-) brain represent mutant cells that are disappearing. Alternatively, the novel β-galactosidase-staining cells are unrelated to NLOT. In this case, NLOT neurons die before P0, or *Tst-1/Oct-6/SCIP* protein is required for its own expression in these cells.

pressed under the control of the *P₀* promotor in transgenic mice (Weinstein et al. 1995). Within the limitations of in situ hybridization, we observe normal levels of *P₀*, *MBP*, *MAG*, and *PMP22* gene expression in *Tst-1/Oct-6/SCIP*-null mice, suggesting that if *Tst-1/Oct-6/*

SCIP does regulate these genes in vivo, its effect is small. Furthermore, *Tst-1/Oct-6/SCIP* mutant mice lack the hypertrophic “onion bulb” neuropathy associated with overexpression of *PMP22* [for review, see Chance and Fischbeck 1994], suggesting that myelin structural genes

Figure 7. Schematic diagram depicting arrest of $(-/-)$ Schwann cells at the ensheathment stage, whereas expression of major myelin genes is normal. Low-level expression of the genes depicted is not shown. *Tst-1/Oct-6/SCIP* expression is transient (Monuki et al. 1990; Scherer et al. 1994; S. Scherer, unpubl.) and precedes the peak of *Krox-20* and myelin gene expression. *Pax3* expression declines before myelination. (SC) Schwann cell; (A) axon.

are not overexpressed in these mice. Because the P_0 - Δ *SCIP* transgenic mice, unlike *Tst-1/Oct-6/SCIP*-null mice, are able to wrap axons, we postulate that under certain circumstances, Δ *SCIP* is likely to act as a transcriptional activator in vivo, perhaps through interactions between its POU domain and other proteins. In muscle development, the DNA-binding domain of either the MADS-box factor myocyte-specific enhancer factor 2 (MEF2) or the helix-loop-helix (HLH) transcription factor myogenin is sufficient for a complex to form that activates target genes (Molkentin et al. 1995). The occasional myelinated axons in *Tst-1/Oct-6/SCIP* mutant mice, which may be analogous to the few myotubes that form in myogenin mutant mice (Hasty et al. 1993; Nabeshima et al. 1993), could be accounted for by the formation of a partial activating complex even in the absence of *Tst-1/Oct-6/SCIP*. Our results suggest that the function of *Tst-1/Oct-6/SCIP* in peripheral myelination is distinct from the regulation of the major myelin structural proteins. The genes that *Tst-1/Oct-6/SCIP* activate or repress are unknown; they could be involved in key aspects of axonal wrapping such as intracellular signaling or membrane biogenesis.

Other transcription factors also have key roles in the development of Schwann cells, and their expression patterns are also diagrammed in Figure 7. *Pax3* is expressed throughout development of the Schwann cell lineage, and in nonmyelinating Schwann cells; it appears to be required for the establishment and maintenance of embryonic Schwann cells, and its expression is down-regulated before myelination (Kioussi et al. 1995). Schwann cells from *Krox-20* mutant mice also fail to wrap axons (Topilko et al. 1994), but these mutant Schwann cells differ from those that lack *Tst-1/Oct-6/SCIP* in two aspects. Occasional myelinating figures are not observed in *Krox-20* mutant Schwann cells and they express reduced levels of P_0 mRNA, in contrast to the apparently normal levels of P_0 mRNA expression in comparably aged *Tst-1/Oct-6/SCIP* mutant mice. Our results suggest that the absence of axonal wrapping in *Krox-20* mutant mice could result from a failure of *Tst-1/Oct-6/SCIP* expression, whereas *Krox-20* independently appears to regulate P_0 mRNA expression. Alternatively, *Tst-1/Oct-6/SCIP* and *Krox-20* are independently regulated, but they cooperate in the activation of axonal wrapping.

Neuronal functions of the *Tst-1/Oct-6/SCIP* gene

In contrast to its dramatic effects on Schwann cell differentiation, deletion of *Tst-1/Oct-6/SCIP* affects only a subset of the neurons that express it. Detailed examination of β -galactosidase marker gene expression reveals no differences between $(+/-)$ and $(-/-)$ mice in the vast majority of *Tst-1/Oct-6/SCIP*-expressing neurons. However, we did observe abnormalities in the position and/or number of phrenic motoneurons in mutant mice. Although this defect may cause the observed reduction in breathing frequency, other respiratory neurons, including those of the nucleus tractus solitarius and the nucleus ambiguus, may also require *Tst-1/Oct-6/SCIP* to obtain a normal differentiated phenotype. In concert with the phrenic motoneuron abnormalities, a failure of lung inflation was observed in neonatal *Tst-1/Oct-6/SCIP* mutant mice. The *Tst-1/Oct-6/SCIP* $(-/-)$ mice therefore provide a useful model for further investigation into the neural control of pulmonary function.

A second neuronal defect in *Tst-1/Oct-6/SCIP* mutant mice involves the mismigration and disappearance of cells of the nucleus of the lateral olfactory tract. These neurons project to the olfactory bulb (de Olmos et al. 1978; Luskin and Price 1983) and the islands of Calleja in the olfactory tubercle (Luskin and Price 1983), and receive axonal projections from the olfactory bulb (Price 1973) and the amygdaloid basal nuclei (Savander et al. 1995); their mislocation in *Tst-1/Oct-6/SCIP* $(-/-)$ mice suggests that these mice may possess deficits in the processing of olfactory information. Many other olfactory neurons express *Tst-1/Oct-6/SCIP* (Alvarez-Bolado et al. 1995; data not shown) but appear unaffected by its deletion. These findings are reminiscent of cell migration defects caused by mutations of the POU domain proteins *Unc-86* (Finney and Ruvkin 1990), *ceh-18* (Greenstein et al. 1994), and *drifter* (Anderson et al. 1995). Notably, nematodes that possess mutations in the *Unc-86* gene are unable to chemotax (Finney and Ruvkin 1990).

Related functions of *Tst-1/Oct-6/SCIP* and other POU domain proteins

The functions of *Tst-1/Oct-6/SCIP* in development and differentiation are likely to overlap those of other POU-

domain proteins, and this may account for some cell types that express Tst-1/Oct-6/SCIP, but appear to be unaffected by its deletion. Oligodendrocytes appear to myelinate normally in *Tst-1/Oct-6/SCIP* $(-/-)$ mice, perhaps because they also express other class III POU proteins [M. Wegner, unpubl.]. In contrast, the only POU-domain proteins known to be expressed in Schwann cells are Tst-1/Oct-6/SCIP and Oct-1 [Kuhn et al. 1991]. Multiple class III POU proteins are coexpressed with Tst-1/Oct-6/SCIP in neurons of cortical layers 2/3 and 5 [Alvarez-Bolado et al. 1995], where no defect has been observed. In the medial habenula, Tst-1/Oct-6/SCIP and the acetylcholine receptor subunit $\alpha 3$ are coexpressed, and Tst-1/Oct-6/SCIP has been reported to activate $\alpha 3$ in vitro [Yang et al. 1994]. However, other POU-domain proteins are expressed in the medial habenula [Alvarez-Bolado et al. 1995], and $\alpha 3$ expression is unaffected in Tst-1/Oct-6/SCIP mutant mice (data not shown).

The target genes of Tst-1/Oct-6/SCIP in Schwann cells may be closely related to the genes that are regulated by POU-domain proteins in the differentiation of other cell types. Disruption of Oct-2 arrests maturation of B cells without altering expression of several genes thought to be regulated by Oct-2 [Corcoran et al. 1993; Feldhaus et al. 1993]. Therefore, the role of Tst-1/Oct-6/SCIP in peripheral myelination may be conceptually similar to that of Oct-2 in the terminal differentiation of B cells, and may reflect the functions of other POU-domain proteins as well.

The results presented here establish a unique role for the Tst-1/Oct-6/SCIP gene product in the regulation of peripheral myelination, and in the differentiation of specific neurons. These data redefine the role of Tst-1/Oct-6/SCIP in the differentiation of Schwann cells, and have implications for the etiology of demyelinating disorders. Some cases of arthrogryposis multiplex congenita with hypomyelinating neuropathy in humans appear to have Schwann cells that are arrested at the ensheathment stage [Charnas et al. 1988; Boylan et al. 1992], suggesting that the etiology of some of these cases could involve mutations of the *Tst-1/Oct-6/SCIP* gene.

Materials and methods

Construction of TKAB', a general vector for creating homologous recombinants by positive/negative selection in ES cells

To create a vector that could be used to generate a variety of knockout constructs that use positive/negative selection, a novel polylinker containing a number of unique rare and common restriction sites was fused to the 3' end of a PGK-tk expression cassette. Mouse sequences and the *neo* gene can be cloned into any of several common or rare sites, and the resulting knockout vector can then be linearized with one of several rare cutting restriction enzymes. To create this vector, pBKSII⁺ was cut with *Asp718* and *XhoI*, filled in, then ligated to the PGK-1-HSV1-tk insert from pGEM7(tk) [M.A. Rudnicki, Massachusetts Institute of Technology, Cambridge] that had been cut with *EcoRI* and *SalI*, then filled in. Most of the remaining polylinker of pBKSII⁺ was subsequently replaced in two steps with oligonucleotides containing the new restriction sites. The

resulting vector, TKAB', is 5.9 kb in length, and consists of the PGK-tk expression cassette followed by *SalI*, *Clal*, *XbaI*, *EcoRI*, *HindIII*, *BamHI*, *XhoI*, *Sse8387*, *Nhe*, *Sfi*-*Pml*, and *NotI* restriction sites.

Construction of Tst-1/Oct-6/SCIP knockout vector

Mouse Tst-1/Oct-6/SCIP genomic sequences were isolated from a J1 mouse ES cell genomic library in phage λ (kindly provided by En Li, Whitehead Institute for Biomedical Research, Cambridge, MA). A 6.3-kb fragment of a 5' Tst-1/Oct-6/SCIP sequence, flanked by the *MscI* site that cuts within the second codon of the mouse Oct-6 sequence [Meijer et al. 1990; Suzuki et al. 1990], was ligated to the 5' *BamHI* site of the β -geo *lacZ*-*neo* fusion gene (P. Soriano and G. Friedrich, Baylor College of Medicine, Houston, TX) after the *MscI* site was modified by ligation of 8-mer *BglII* linkers (NE Biolabs). The first two amino acids of Tst-1/Oct-6/SCIP and β -geo are identical, and ligation of the *BglII* and *BamHI* sites fused the codons for these amino acids to the third codon of β -geo. Subsequently, sequences 3' to the *Clal* site in the *LacZ* portion of β -geo were replaced with unfused *LacZ* and pMC1-*neo* sequences that were obtained from the plasmid pC19H- β -galpAneo B-B 1 [O. Chisaka and M. Capecchi, University of Utah, Salt Lake City]. A 4.0-kb *SacII*-*XhoI* fragment that contains Tst-1/Oct-6/SCIP 3' coding and noncoding sequences was ligated to the 3' end of the pMC1-*neo* gene. The resulting sequences were cloned into the vector TKAB'. A clone that contains Tst-1/Oct-6/SCIP 5' sequences adjacent to the *tk* gene was used as the Tst-1/Oct-6/SCIP knockout vector, after being linearized with *XhoI*.

Production of mutant mice

Mutant ES cells and mice were generated using standard techniques [Hogan et al. 1994], using J1 ES cells (kindly provided by En Li), which were grown in the presence of 1000 U/ml of leukemia inhibitory factor on mouse embryonic fibroblast feeder cells. Recombinant cell lines were selected using neomycin (GIBCO), and Gancyclovir (kindly provided by Syntex). Three independent heterozygous ES cell lines were detected by Southern analysis, using a 5' probe that consists of 0.4 kb between the *BglII* and *NheI* sites that are located ~6 kb 5' to the Tst-1 promoter, and a 3' probe that consists of a 0.6-kb *XhoI*-*BglII* fragment located 4 kb 3' to the Tst-1 POU domain. Both probes are located outside of the knockout vector. Cells from all three lines were injected into C57BL/6J blastocysts. Chimeras derived from one line produced surviving agouti offspring when mated to C57BL/6 females, and were backcrossed either to C57BL/6J females, or to 129/Sv agouti females (kindly provided by Jeff Mann, Beckman Research Institute of City of Hope, Duarte, CA).

Genotype analysis of mutant mice

Initial litters from heterozygous intercrosses were analyzed by southern analysis of DNA extracted from the pups. Subsequently, the litters were analyzed by PCR, using the following primers: Neokopcr1, 5'-AGAGGCTATTTCGGCTATGAC-TGGGCA-3'; Neokopcr2, 5'-CCACACCCAGCCGGCCA-CAGTCGATGA-3'; Tstkopcr3, 5'-CACTGCACGAGGACG-GCCAC-3'; and Tstkopcr4, 5'-TCGAGCGCGCCTTTGACACC-3'. Ten-milliliter reactions that were 10 μ M Tris (pH8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μ M each dNTP, 10% DMSO, contained 0.1 μ g of each of the four primers, 0.2 μ l of *Taq* polymerase, and 0.5 μ l DNA template were cycled in a Perkin-Elmer 9600 PCR machine at 94°C, 30 sec; 65°C, 30 sec; 72°C, 30

sec; for 40 cycles, followed by 5 min, 72°C. +/+ , +/- , -/- , and no DNA controls were used with each set of reactions. Reaction products of 593 bp for the *neo* primers and 497 bp for the Tst-1 primers were visualized by ethidium bromide staining after electrophoresis on 1.2% TBE-agarose gels.

Microscopy of myelin figures

Mice were perfused with 3.2% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), after which the spinal cords and sciatic nerves were removed and placed in fresh fixative overnight at 4°C. The tissues were osmicated, dehydrated, infiltrated, and embedded in epoxy resin. Semithin sections were stained with methylene blue-azure II-basic fuchsin. For electron microscopy, tissues were prepared as described above. Thin sections were stained with lead citrate and uranyl acetate, before electron microscopy.

Immunohistochemistry

Tst-1/Oct-6/SCIP immunoreactivity was revealed with diaminobenzidine using an ABC kit (Vector) in tissues that typically were fixed in 4% paraformaldehyde, frozen, sectioned, and incubated with a 1:1000 dilution of a rabbit polyclonal antiserum raised against a full-length Tst-1/Oct-6/SCIP protein that had been expressed from baculovirus, and kindly provided by M. Wegner (Zentrum für Molekulare Neurobiologie, Hamburg, Germany) and S. Rhodes (IUPUI, Indianapolis).

Sciatic nerves were fixed in 4% paraformaldehyde, infiltrated with 20% sucrose, and embedded in OCT. Transverse sections were mounted on glass slides, blocked in 5% fish skin gelatin, incubated overnight in primary antibody at 4°C, then visualized with fluorescein-conjugated donkey anti-rabbit antiserum (Jackson ImmunoResearch) using a Leica scanning laser confocal microscope. The following rabbit antisera (and dilutions) were used: anti-laminin (1:500), anti-P₀ (1:250), anti-MAG (1:250), and anti-periaxin (1:500), which were obtained from Sigma, Dr. D. Colman (Mt. Sinai School of Medicine, New York, NY), Dr. J. Salzer (New York University School of Medicine, New York), and Dr. P. Brophy (University of Edinburgh, Scotland), respectively.

β-galactosidase staining was performed as described (Hogan et al. 1994).

In situ hybridization

Tissues were fixed in formalin, frozen in a 1:1 mixture of OCT and aquamount, sectioned at 20 μm, and hybridized as described (Simmons et al. 1989). The following antisense probes were used: MAG, a 900-bp *Eco*RI fragment of rat-coding region; P₀, 1.7-kb rat cDNA; MBP, 660-bp of rat-coding sequence. p75 NGFR, 350 bp of mouse exon 3; and Tst-1/Oct-6/SCIP, 0.7 kb of sequence between the AlwN1 and Pst-1 sites (Meijer et al. 1990; Suzuki et al. 1990).

Breathing analysis

Respiration of mouse pups was measured using a plethysmograph that consisted of a pneumotachograph (A. Fleisch Co.) attached to a chamber in which the mouse pup breathed. A mixture of O₂, CO₂ and N₂ flowed around the pup's nose and mouth, which protruded from the chamber through a hole in a piece of latex rubber. The gas mixture was adjusted using a rotameter, and its composition was monitored by continuous sampling using a mass spectrometer. Data were collected on a chart recorder, and on an IBM PC, using Labdat 7.0 software.

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Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration.

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